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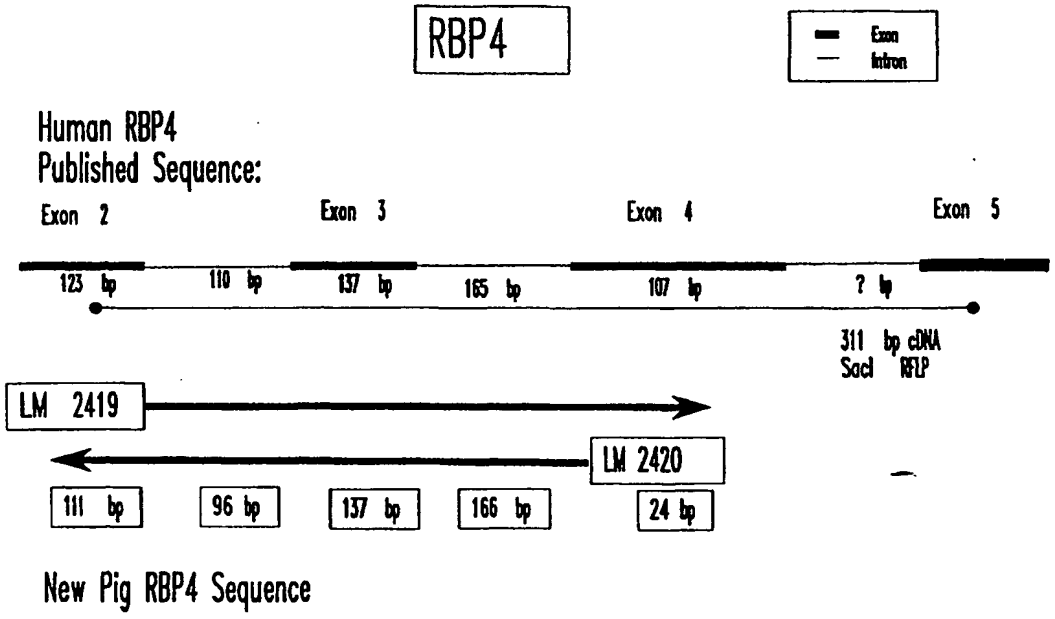

(57) Abstract

Disclosed herein are genetic markers for favorable reproductive traits in animals such as litter size, and weaning weight. Methods for identifying such markers, and methods of screening animals to determine those more likely to produce favorable reproductive traits and preferably selecting those animals for future breeding purposes. The marker is based upon the presence or absence of certain polymorphisms in the pig reproductive gene, retinol binding protein 4.

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TITLE: RETINOL BINDING PROTEIN 4 AS A GENETIC MARKER FOR INCREASED LITTER SIZE

FIELD OF THE INVENTION

This invention relates generally to the detection of genetic differences for reproductive efficiency among animals. More particularly the invention relates to genetic markers which have been identified in several genes indicative of heritable phenotypes associated with improved reproductive traits. Methods and compositions for use of these markers in genotyping of animals and selection are also disclosed.

BACKGROUND OF THE INVENTION

Reproductive efficiency, particularly as it relates to litter size, is the major limiting factor in the efficient production of pork as well as most other livestock animals. Genetic variability exists for several reproductive measures. Average litter size among breeds pigs varies from 4-16 pigs per litter. Mean age at puberty varies from 3 to 7 months of age. This genetic variability within breeds suggests that genetic improvement in reproduction is possible. The number of pigs born alive in the United States averages approximately 9.5 pigs per litter. Heritability for litter size is low (10%-15%), and standard genetic methods of selecting breeding females on the basis of past litter size have not been effective. Therefore, there is a need for an approach that deals with selection for reproductive traits at the cellular or DNA level.

Chinese breeds are known for reaching puberty at an early age and for their large litter size. American breeds are known for their greater growth rates and leanness. Thus, it would be desirable to combine the best characteristics of both types of breeds, thereby improving the efficiency of U.S. pork production. These efforts would be greatly assisted by the discovery of genes or genetic markers that are associated with improved reproductive traits such as increased litter size in pigs.

RFLP analysis has been used by several groups to study pig DNA. Jung et al., Theor. Appl. Genet., 77:271-274 (1989), incorporated herein by reference, discloses the use of RFLP techniques to show genetic variability between two pig breeds. Polymorphism was demonstrated for swine leukocyte antigen (SLA) Class I genes in these breeds. Hoganson et al., Abstract for Annual Meeting of Midwestern Section of the American Society of Animal Science, March 26-28, 1990, incorporated herein by reference, reports on the polymorphism of swine major histocompatibility complex (MHC) genes for Chinese pigs, also demonstrated by RFLP analysis. Jung et al. Animal Genetics, 26:79-91 (1989), incorporated herein by reference, reports on RFLP analysis of SLA Class I genes in certain boars. The authors state that the results suggest that there may be an association between swine SLA/MHC Class I genes and production and performance traits. They further state that the use of SLA Class I restriction fragments, as genetic markers, may have potential in the future for improving pig growth performance.

Further, United States Patent 5,550,024 to Rothschild et al. discloses a polymorphism in the pig estrogen receptor gene which is associated with larger litter size, the disclosure of which is incorporated herein by reference.

Another pig hormone related to beneficial reproductive traits is Prolactin. Prolactin (PRL) is an anterior pituitary peptide hormone involved in many different endocrine activities, but is essential for reproductive success. Use of polymorphic loci in the prolactin receptor gene as markers for increased litter size is described and disclosed in United States Patent Application Serial No. 08/812,208, the disclosure of which is hereby incorporated by reference.

The present invention provides genetic markers, based upon the discovery of polymorphisms in the RBP4 gene, which relate to improved reproductive traits such as litter size and number born alive. This will permit genetic typing of pigs for their reproductive genes and for determination of the relationship of specific genes and markers to reproductive traits. It will also permit the identification of individual males and females that carry beneficial genotypes. In the case of females it would permit that a female would be

expected to produce a litter size larger than the average earlier than average or healthier than average for their breed, or in the case of males, for their female offspring to have the beneficial traits. Thus, the markers will be selection tools in breeding programs to develop lines and breeds that produce litters with favorable reproductive phenotypes.

It is an object of the invention to provide a method of screening pigs to determine those more likely to produce larger litters.

Another object of the invention is to provide a method for identifying genetic markers for reproductive traits such as pig litter size.

A further object of the invention is to provide genetic markers for pig litter size.

Yet another object of the invention is to provide a kit for evaluating a sample of pig DNA for specific genetic markers associated with favorable reproductive traits.

Additional objects and advantages of the invention will be set forth in part in the description that follows, and in part will be obvious from the description, or may be learned by the practice of the invention. The objects and advantages of the invention will be attained by means of the instrumentality's and combinations particularly pointed out in the appended claims.

SUMMARY OF THE INVENTION

To achieve the objects and in accordance with the purpose of the invention, as embodied and broadly described herein, the present invention provides a method for screening pigs and other animals to determine those more likely to have beneficial reproductive phenotypes such as a larger litter, when bred or to select against pigs which have alleles indicating unfavorable phenotypes.

As used herein "larger litters" means a significant increase in litter size above the mean of a given population. As used herein the term "reproductive trait" shall include any trait which is indicative of improved reproduction

efficiency including but not limited to testicular size, sperm volume, sperm concentration, sperm quality, libido, breeding aggressiveness, litter size, number born alive, litter birth weight, number weaned, age at puberty, weaning to oestrus interval, farrowing interval, ovulation rate, uterine capacity, and embryo survival.

As used herein the term "reproductive gene" shall mean any gene which encodes a gene product which, upon expression, influences either favorably or negatively a reproductive trait. Examples of such genes include but are not limited to the estrogen receptor gene, the prolactin receptor gene and the retinol binding protein 4 gene and other genes disclosed and described herein.

Thus, the present invention provides a method for screening pigs to determine those more likely to produce beneficial reproductive traits such as larger litters, and/or those less likely to produce smaller litters, which method comprises the steps 1) obtaining a sample of genomic DNA from a pig or other animal; and 2) analyzing the genomic DNA obtained in 1) to determine which allele(s) of the retinol binding protein 4 is/are present. Briefly, the sample of genetic material is obtained and is analyzed to determine the presence or absence of a polymorphism in a gene that is correlated with a desirable reproductive trait.

In a preferred embodiment the polymorphism is a restriction fragment length polymorphism and the assay comprises identifying the reproductive gene from isolated genetic material; exposing the gene to a restriction enzyme that yields restriction fragments of the gene of varying length; separating the restriction fragments to form a restriction pattern, such as by electrophoresis or HPLC separation; and comparing the resulting restriction fragment pattern from an animal reproductive gene that is either known to have or not to have the desired marker. If an animal tests positive for the marker, such animal can be considered for inclusion in the breeding program. If the animal does not test positive for the marker genotype the animal can be culled from the group and otherwise used.

In a most preferred embodiment the gene of a fragment thereof is isolated by the use of primers and DNA polymerase to amplify a specific region of the gene which contains the polymorphism. Next the amplified region is either directly separated or sequenced or is digested with a restriction enzyme and fragments are again separated. Visualization of the separated fragments or RFLP pattern is by simple staining of the fragments, or by labeling the primers or the nucleoside triphosphates used in amplification.

In another embodiment, the invention comprises a method for identifying a genetic marker for reproductive traits such as litter size in a particular population. Male and female animals of the same breed or breed cross or similar genetic lineage are bred, and the number of offspring produced by each female is determined. A polymorphism in the reproductive gene of each animal is identified and associated with the desired reproductive trait. Preferably, PCR-RFLP analysis is used to determine the polymorphism.

It is also possible to establish linkage between specific alleles of alternative DNA markers and alleles of DNA markers known to be associated with a particular gene (e.g. the reproductive genes discussed herein), which have previously been shown to be associated with a particular trait. Thus, in the present situation, taking a particular reproductive gene, it would be possible, at least in the short term, to select for pigs or other animals likely to produce larger litters, or alternatively against pigs likely to produce smaller litters, indirectly, by selecting for certain alleles of a particular reproductive gene associated marker through the selection of specific alleles of alternative chromosome markers. For example markers known to be linked to retinol binding protein 4 gene on porcine chromosome 14 includes S0007, S0116, and SW210, which are all microsatellites.

The invention further comprises a kit for evaluating a sample of DNA for the presence in genetic material of a desired genetic marker located in the reproductive gene indicative of the inheritable reproductive trait such as large litter size. At a minimum, the kit is a container with one or more reagents that identify a polymorphism in the RBP4 gene. Preferably, the reagent is a

set of oligonucleotide primers capable of amplifying a fragment of the selected reproductive gene that contains a polymorphism. Preferably, the kit further contains a restriction enzyme that cleaves the reproductive gene in at least one place, allowing for separation of fragments and detection of polymorphic loci.

The accompanying figures, which are incorporated herein and which constitute a part of this specification, illustrate one embodiment of the invention and, together with the description, serve to explain the principles of the invention.

DESCRIPTION OF THE FIGURES

Figure 1 is a schematic depicting the expected fragment pattern using the second PCR protocol and primers in Example 5.

Figure 2 is a schematic depicting a comparison of the human published retinol binding protein 4 sequence and the new pig retinol binding protein 4 sequence.

DETAILED DESCRIPTION OF THE INVENTION

Reference will now be made in detail to the presently preferred embodiments of the invention, which together with the following examples, serve to explain the principles of the invention.

The invention relates to genetic markers for beneficial reproductive traits such as litter size in pigs and other animals. It provides a method of screening animals to determine those more likely to produce earlier, healthier, or larger litters when bred by identifying the presence or an absence of a polymorphism in certain reproductive genes (namely the RBP4 gene) that are correlated with these reproductive traits.

Thus, the invention relates to genetic markers and methods of identifying those markers in a pig or other animal of a particular breed, strain, population, or group, whereby the female is more likely to produce a litter that is significantly larger (number) is healthier, is earlier to mature, etc. above the mean for that particular breed, strain, population, or group. Any method of

identifying the presence or absence of this marker may be used, including for example single-strand conformation polymorphism (SSCP) analysis, RFLP analysis, heteroduplex analysis, denaturing gradient gel electrophoresis, and temperature gradient electrophoresis, ligase chain reaction or even direct sequencing of the reproductive gene and examination for the certain recognition patterns.

Other possible techniques include non-gel systems such as TaqMan™ (Perkin Elmer). In this system oligonucleotide PCR primers are designed that flank the mutation in question and allow PCR amplification of the region. A third oligonucleotide probe is then designed to hybridize to the region containing the base subject to change between different alleles of the gene. This probe is labeled with fluorescent dyes at both the 5' and 3' ends. These dyes are chosen such that while in this proximity to each other the fluorescence of one of them is quenched by the other and cannot be detected. Extension by Taq DNA polymerase from the PCR primer positioned 5' on the template relative to the probe leads to the cleavage of the dye attached to the 5' end of the annealed probe through the 5' nuclease activity of the Taq DNA polymerase. This removes the quenching effect allowing detection of the fluorescence from the dye at the 3' end of the probe. The discrimination between different DNA sequences arises through the fact that if the hybridization of the probe to the template molecule is not complete, i.e. there is a mismatch of some form, the cleavage of the dye does not take place. Thus only if the nucleotide sequence of the oligonucleotide probe is completely complementary to the template molecule to which it is bound will quenching be removed. A reaction mix can contain two different probe sequences each designed against different alleles that might be present thus allowing the detection of both alleles in one reaction.

The use of RFLPs is the preferred method of detecting the polymorphism most preferred PCR-RFLP analysis. However, since the use of RFLP analysis depends ultimately on polymorphisms and DNA restriction sites along the nucleic acid molecule, other methods of detecting the

polymorphisms can also be used. Such methods include ones that analyze the polymorphic gene product and detect polymorphisms by detecting the resulting differences in the gene product.

RFLP analysis in general is a technique well-known to those skilled in the art. See, for example, U.S. Patents 4,582,788 issued April 15, 1986 to Erlich and 4,666,828 issued May 19, 1987 to Gusella, 4,772,549 issued September 20, 1988 to Frossard, and 4,861,708 issued August 29, 1989 to Frossard, the disclosures of which are incorporated herein by reference. Broadly speaking, the technique involves obtaining the DNA to be studied, digesting the DNA with restriction endonucleases, separating the resulting fragments, and detecting the fragments of various genes.

In the present invention, a sample of genetic material is obtained from an animal. Samples can be obtained from blood, tissue, semen, etc. Generally, peripheral blood cells are used as the source, and the genetic material is DNA. A sufficient amount of cells are obtained to provide a sufficient amount of DNA for analysis. This amount will be known or readily determinable by those skilled in the art as explained in the material incorporated herein. The DNA is isolated from the blood cells by techniques known to those skilled in the art.

Next the region containing the polymorphism is amplified by the use of primers and standard techniques, such as the polymerase chain reaction. This technique is described in U.S. Patents 4,683,195, issued July 28, 1987 to Mullis et al., 4,683,202, issued July 28, 1987 to Mullis, 4,800,159 issued January 24, 1989 to Mullis, et al., 4,889,818 issued December 26, 1989 to Gelfand, et al., and 4,902,624, issued February 20, 1990 to Clumbus, et al., all of which are incorporated herein by reference. The selection of primers is discussed in the references mentioned and incorporated herein. The primers should amplify the region containing the polymorphism. Several primers for specific polymorphisms are disclosed herein. Other such primers are designable by those of skill in the art combined with the teachings herein, and are intended to be encompassed by the invention.

The isolated DNA is then analyzed and optionally digested with a restriction endonuclease that cleaves or cuts DNA hydrolytically at a specific nucleotide sequence, called a restriction site. Such endonucleases, also called restriction enzymes, are well-known to those skilled in the art. For the present invention, one should be chosen that cleaves the selected reproductive gene in at least one place, producing at least two fragments of the gene. A determination is made as to whether or not any such fragments are polymorphic and if any polymorphism (RFLP) is associated with a desired reproductive trait such as litter size by techniques known in the art in conjunction with the teachings contained herein. The amount of such enzyme to be added to the sample containing the pig DNA and the other appropriate conditions for treating the sample will be readily determinable to persons skilled in the art, given the teachings contained herein.

The restriction fragments are then analyzed by known techniques that generally involve either the separation of the fragments and visualization by staining or subsequent blotting and hybridization to obtain a particular pattern or the determination of different sizes of the fragments. The latter permits the identification of one or more fragments (markers) for increased litter size. The preferred separation technique is gel electrophoresis.

In this technique, the digested fragments are separated in a supporting medium by size under the influence of an applied electric field. Gel sheets or slabs, such as agarose or agarose-acrylamide, are typically used as the supporting medium. The sample, which contains the restriction fragments, is added to one end of the gel. One or more size markers are run on the same gel as controls to permit an estimation of the size of the restriction fragments. This procedure generally permits a degree of resolution that separates fragments that differ in size from one another by as little as 100 base pairs.

In alternative embodiments, the fragments are denatured and transferred physically from the gel onto a solid support, preferably a nylon membrane, by contacting the gel with the filter in the presence of appropriate reagents and under appropriate conditions that promote the transfer of the

DNA. Such reagents and conditions are well-known to those skilled in the art. Thus, the relative positions of the DNA fragments resulting from the separation procedure are maintained.

The next step involves the detection of the various categories of sizes of the fragments or, alternatively, the detection of a fragment of a particular size. The latter may be of particular interest because it is a genetic marker associated with a desired reproductive trait. This is preferably accomplished via staining of the fragments with ethidium bromide or the like.

An alternative technique is the use of a hybridization probe. Such a probe is an oligonucleotide or polynucleotide that is sufficiently complementary or homologous to the fragments to hybridize with them, forming probe-fragment complexes. Preferably, the probe is a cDNA probe. The oligonucleotide or polynucleotide is labeled with a detectable entity. This permits the detection of the restriction fragments, to which the probes are hybridized. The probes are labeled by standard labeling techniques, such as with a radiolabel, enzyme label, fluorescent label, biotin-avidin label, and the like. See U.S. patent nos. 4,711,955 issued December 8, 1987 to Ward et al. and 4,868,103 issued September 19, 1989 to Stavrianopoulos et al., both of which are incorporated herein by reference.

The probes are contacted with the nylon membrane that contains the restriction fragments for a sufficient period of time and under appropriate hybridizing conditions for the probes to hybridize to the fragments. The filter is then preferably washed to remove unbound probes and other unwanted materials.

The probe-fragment complexes, which are bound to the filter, are then detected by known techniques. For example, if the probe has been radioactively labeled (^{32}P), detection involves contacting the nylon membrane paper with a piece of radiosensitive film. Following an appropriate exposure period, the fragments of interest, including control fragments, are visualized.

The detection step provides a pattern, resulting from the separation of the fragments by size. Comparison of these fragments with control fragments

of known size that have also been run on the same gel permits the estimation of the size of the various groups of fragments. The various polymorphisms in the reproductive genes are then determined by comparison of the patterns produced by similar analysis of DNA from a number of different pigs. For some of the individual animals, the patterns will differ from the usual pattern produced by most of the other animals. This will be due to one or more restriction fragment length polymorphisms, i.e., restriction fragments of a different length produced by the endonuclease that cuts the reproductive gene. This indicates different base pair sequences in such pigs.

Once a particular RFLP has been identified, i.e., a restriction fragment of a particular length, a probe to this fragment may be constructed by the use of known techniques. This permits alternative and faster formats for detecting such polymorphism. For example, once the DNA is digested, a sandwich hybridization format can be used. Such an assay is disclosed in US. Patents 4,486,539 issued December 4, 1984 to Ranki, et al., and 4,563,419 issued January 7, 1986 to Ranki, et al., both of which are incorporated herein by reference. The sample is brought into contact with a capture probe that is immobilized on a solid carrier. The probe binds the fragment. The carrier is then washed, and a labeled detection probe is added. After additional washing, the detection probe is detected, thereby demonstrating the presence of the desired fragment.

In yet another embodiment, once the RFLP pattern has been determined or a particular polymorphic fragment has been determined, it is compared to a second, known RFLP pattern or fragment that is correlated with increased litter size. This second pattern or fragment has also been determined from the reproductive gene, using the same restriction endonuclease as the first and the same probe or an equivalent thereof under the same conditions.

In an alternative embodiment of the invention, the restriction fragments can be detected by solution hybridization. In this technique, the fragments are first hybridized with the probe and then separated. The separated probe-

fragment complexes are then detected as discussed above. Generally, such complexes are detected on the gel without transfer to filter paper.

In a most preferred embodiment the polymorphism is detected by PCR amplification without any probe. This procedure is known to those of skill in the art and is disclosed in U.S. Patents 4,795,699 entitled "DNA Polymerase" and U.S. Patent 4,965,188 "Process for Amplifying, Detecting, and/or Cloning Nucleic Sequences Using a Thermostable Enzyme" both of which are incorporated herein by reference.

For this procedure primers are constructed to amplify the region in which the polymorphism lies. Accordingly primers which are preferably 4-30 bases are designed based upon the sequence surrounding the polymorphism including a forward 5', primer and a reverse or anti-sense primer 3' of the polymorphism. The primers need not be the exact complement, and substantially equivalent sequences are also acceptable. A DNA polymerase is then added such as Taq polymerase (many such polymerases are known and commercially available) in the presence of the four nucleoside triphosphates and often a buffering agent. Detection is facilitated by simple staining, such as with ethidium bromide, of separated products to detect for predicted sizes based upon the length of the region amplified. Reaction times, reagents, and design of primers are all known to those of skill in the art and are discussed in the patents incorporated herein by reference. Further PCR amplification may be used in combination with Single Strand Confirmation Polymorphism (SSCP). See Detection of Polymorphism, of Human DNA by Gel Electrophoresis as Single-Strand Conformation Polymorphisms, Orita et al, PNAS 86(8) Apr. 1989 (2766-70); and Lessa et al. Mol Ecol 2(2) p. 119-29 Apr 1993 "Screening Techniques for Detecting Allelic variation in DNA Sequences" which are incorporated by reference.

Although the methods described herein may be in terms of the use of a single restriction enzyme and a single set of primers, the methods are not so limited. One or more additional restriction enzymes and/or probes and/or primers can be used, if desired. Additional enzymes, constructed probes and

primers can be determined through routine experimentation, combined with the teachings provided and incorporated herein.

Genetic markers for reproductive genes or particularly for the RBP4 gene or other genes linked therewith are determined as follows. Male and female animals of the same breed or breed cross or derived from similar genetic lineages are mated. The number of offspring with the beneficial reproductive trait is determined. For litter size the number of offspring produced by each female is determined. RFLP analysis of the parental DNA is conducted as discussed above in order to determine polymorphisms in the selected reproductive gene of each animal. The polymorphisms are associated with the traits. At least 20 and preferably at least 40 females are used in making these determinations. The number of times each female produces a litter (i.e., the parity) is at least 1 time. Preferably, the cycle of breeding and giving birth is repeated at least 2 times and most preferably 3 times.

When this analysis is conducted and the polymorphism is determined by RFLP or other analysis amplification primers may be designed using analogous human or other closely related animal known sequences. The sequences of many of the reproductive genes have high homology. Primers may also be designed using known gene sequences as exemplified in Genbank or even designed from sequences obtained from linkage data from closely surrounding genes. According to the invention sets of primers have been selected which identify regions in polymorphic reproductive genes. The polymorphic fragments have been shown to be alleles, and each was shown to be associated with beneficial reproductive traits, such as increased litter size, for various breeds. Often genotype associated with this trait alternates for different breeds. This outcome is similar to the situation disclosed in U.S. patent 5,374,523 entitled "Allelic variants of Bovine Somatotropin gene: Genetic marker for Superior Milk Production in Bovine" where the inventor found an allelic polymorphism is the somatotropin gene and one allelic form was beneficial for jersey cows and the alternate form was beneficial for Holstein cows.

The reagents suitable for applying the methods of the invention may be packaged into convenient kits. The kits provide the necessary materials, packaged into suitable containers. At a minimum, the kit contains a reagent that identifies a polymorphism in the selected reproductive gene that is associated with a reproductive trait such as increased litter size. Preferably, the reagent is a PCR set (a set of primers, DNA polymerase and 4 nucleoside triphosphates) that hybridize with the reproductive gene or a fragment thereof. Preferably, the PCR set and a restriction enzyme that cleaves the reproductive gene in at least one place are included in the kit. Preferably, the kit further comprises additional means, such as reagents, for detecting or measuring the detectable entity or providing a control. Other reagents used for hybridization, prehybridization, DNA extraction, visualization etc. may also be included, if desired.

The methods and materials of the invention may also be used more generally to evaluate animal DNA, genetically type individual animals, and detect genetic differences in animals. In particular, a sample of genomic DNA may be evaluated by reference to one or more controls to determine if a polymorphism in the reproductive gene is present. Preferably, RFLP analysis is performed with respect to the reproductive gene, and the results are compared with a control. The control is the result of a RFLP analysis of the reproductive gene of a different animal where the polymorphism of the reproductive gene is known. Similarly, the reproductive genotype of an animal may be determined by obtaining a sample of its genomic DNA, conducting RFLP analysis of the reproductive gene in the DNA, and comparing the results with a control. Again, the control is the result of RFLP analysis of the reproductive gene of a different animal. The results genetically type the pig by specifying the polymorphism in its selected reproductive gene. Finally, genetic differences among animals can be detected by obtaining samples of the genomic DNA from at least two animals, identifying the presence or absence of a polymorphism in the reproductive gene, and comparing the results.

These assays are useful for identifying the genetic markers relating to litter size, as discussed above, for identifying other polymorphisms in the reproductive gene that may be correlated with other characteristics, and for the general scientific analysis of genotypes and phenotypes.

The genetic markers, methods, and kits of the invention are also useful in a breeding program to improve litter size in a breed, line, or population of animals. Continuous selection and breeding of animals that are at least heterozygous and preferably homozygous for a polymorphism associated with a beneficial reproductive trait such as increased litter size would lead to a breed, line, or population having higher numbers of offspring in each litter of the females of this breed or line. Thus, the markers are selection tools.

The examples and methods herein disclose certain reproductive genes which have been identified to have a polymorphism which is associated either positively or negatively with a beneficial reproductive trait (litter size) that will have an effect on reproductive efficiency of that animal. The identification of the existence of a polymorphism within a gene (RBP4) is often made by a single base alternative that results in a restriction site in certain allelic forms. A certain allele, however, as demonstrated and discussed herein may have a number of base changes associated with it that could be assayed for which are indicative of the same polymorphism. Further, other genetic markers or genes may be linked to the polymorphisms disclosed herein so that assays may involve identification of other genes or gene fragments but which ultimately rely upon genetic characterization of animals for the same polymorphism. Any assay which sorts and identifies animals based upon the allelic differences disclosed herein are intended to be included within the scope of this invention. One of skill in the art, once a polymorphism has been identified and a correlation to a particular trait proven, will understand that there are an infinite number of ways to genotype animals for this polymorphism. The design of these such alternative tests merely represent optimization of parameters known to those of skill in the art and are intended to be within the scope of this invention as fully described herein.

According to the invention a polymorphism in the RBP4 gene has been identified which is associated with increased litter size. Retinol-binding protein 4 (RBP4) is expressed during blastocyst elongation and has been postulated to transport and regulate the amount of retinol received by the fetuses. According to the invention a polymorphism has been identified and RBP4 has been mapped. SacI digests hybridized with RBP4 revealed a polymorphism with two alleles and the linkage analysis found significant linkages with several loci on pig chromosome 14. Further refinement includes 2 PCR tests with primers for detection of a MSP I polymorphism showing a difference between homozygous genotypes of 1.05 pigs per litter.

It is to be understood that the application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. The examples of the products and processes of the present invention appear in the following examples which are not intended to limit the scope or teachings of the invention.

EXAMPLE 1

LINKAGE MAPPING OF THE RETINOL-BINDING PROTEIN 4 (RBP4) GENE TO PORCINE CHROMOSOME 14

Map positions: Loci order of a portion of the distal end of Chromosome (Chr) 14: -ACTN2-1.7-ACTA1-2.7-PLAU-0-SW210-8.2-S0169-9.9-S0072-11.1-S0007-7.3-RBP4-16.2-S0116-20.0-Sw761-36.1-S0015.

Method of mapping: Six three-generation PiGMaP families of Meishan x Large White and European Wild Boar x Large White pigs. Archibald, A. et al., 1995. Mamm. Genome 6:157-175.

Molecular reagents: The porcine RBP4 gene probe was obtained by RT-PCR amplification of a 311-bp fragment from day 12 porcine blastocysts with primers designed based on pig cDNA sequences. The 5' primer (5'-TTCCGAGTCAAAGAGAACTTCG-3' SEQ ID NO:1) represents nucleotides 79-100, and the 3' primer (5'-TCATAGTCCGTGTCGATGATCC-3' SEQ ID NO:2) represents nucleotides 368-389. Trout, W., et al. 1991, Mol. Endocrinol.

5:1533-1540. Amplified product was purified and radiolabeled with ^{32}P by random priming.

Allele detection: A SacI polymorphism was detected in pig genomic DNA by hybridization of Southern blots with the labelled 311 bp pig RBP4 fragment at a final wash stringency of 0.7 x SSC and 0.2% SDS at 65 C. Autosomal Mendelian inheritance was observed in two polymorphic fragments detected at 12.1 kb and 7.8 kb. Sixty-two unrelated pigs from eight breeds were genotyped for RBP4. Frequencies of the 12.1-kb fragment were 0.55 in Landrace (n = 10), 0.75 in Duroc (n = 10), 1.0 in Yorkshire (n = 5), 0.50 in Chester White (n = 4), 0.59 in Large White (n = 11), 1.0 in Hampshire (n = 5), 0.80 in Meishan (n = 15), and 1.0 in Wild Boar (n = 2).

Previously identified homologs: Human RBP4 maps of 10q23-24 Rocchi, M., et al. 1989, Somat. Cell Mol. Genet. 15:185-190, and mouse Rbp-4 is localized to the distal end of Chr 19 Chainani, M., et al., 1991, Genomics 9:376-379.

Discussion: Retinol-binding protein is a major secretory product of the pig conceptus prior to implantation. Increased production of RBP4 during the rapid morphological developmental period of pig blastocyst elongation, which is a critical period for embryonic survival, suggests that RBP4 may be an interesting candidate gene for investigation of QTL for reproduction in pigs.

Linkage analysis was performed with the CRIMAP version 2.4 software package. The two-point linkage analysis produced significant lod scores (>3.0) of RBP4 with loci S0007, S0116, and SW210 on porcine Chr 14 (Table 1). The order of the loci on our map is in agreement with the new loci arrangement of the PiGMaP map of Chr 14 according to Kapke and associates, (Kapke, P., et al., 1995, Anim. Genet., in press) with the exception of the rearrangement of loci S0007 and S0072. The addition of RBP4 to the revised PiGMaP map further increases the sex-averaged map length from 193 cM to 202 cM. Placement of RBP4 on Chr 14 strengthens the homology between porcine Chr 14 and human Chr 10. Johansson, M., et al., 1995, Genomics, 25:682-690.

Table 1. Results of the two-point linkage analysis for RBP4

Marker 1	Marker 2	Recombination fraction	Lod score
RBP4	S0007	0.07	17.02
RBP4	S0116	0.21	4.63
RBP4	SW210	0.27	3.50

EXAMPLE 2

ASSOCIATION OF POLYMORPHISM WITH LITTER SIZE

Partial cDNA fragments ofthe RBP4 gene was used for RFLP detection on Southern membranes containing digested reference family DNA. SacI membranes probed with RBP4 showed two allelic fragments at 12.1 kb and 7.8 kb. RBP4 was mapped by linkage analysis to chromosome 14. To illustrate effects of this gene, two French Large White lines were genotyped at these loci. The first line consisted of French hyperprolific (LWH) pigs (32 sows with 216 litter records). The second line consisted of French control (LW) pigs (27 sows with 242 litter records). The average additive effect of the gene was estimated as the linear regression of litter size on genotype. RBP4 had an additive gene effect of 0.52 ± .30 in LWH and .45 ± .43 in LW for the 7.8 kb allele. The allelic substitutive effect for litter size ranged from 5 to 17% of the phenotypic STD.

EXAMPLE 3 (RBP4)

PCR-RFLP Test for MSPI polymorphism

Based on the identification of polymorphism in the RBP4 gene a PCR test was developed.

Primers

LM2419 5'-GAGCAAGATGGAATGGGTT-3' SEQ ID NO:4

LM2420 5'-CTCGGTGTCTGTAAAGGTG-3' SEQ ID NO:5

PCR conditions:	25 μL reaction
Mix 1:	
10X Promega Buffer	2.5 μL
25 mM $MgC1_2$	1.5 μL
10 mM dNTP's (Boehringer Mannheim)	0.5 μL
16 pMol LM2419 (100 ng/μL)	0.5 μL
16 pMol LM2420 (100 ng/μL)	0.5 μL
dd sterile H_2O	12.5 μL
25 ng genomic DNA (12.5 ng/μL)	2.0 μL
Taq Mix:	
dd sterile H_2O	4.9 μL
0.6 U Taq Polymerase (Promega)	0.12 μL

Combine 18 μL of Mix 1 and DNA in reaction tube. Overlay with Mineral Oil. Preheat on thermal cycler at 85°C. Add 5 ul of Taq Mix. Run the following PCR program:

Step 1.	93°C 3 min
Step 2.	93°C 30 sec
Step 3.	56°C 45 sec
Step 4.	72°C 45 sec
Step 5.	Go to step 2 for 39 more cycles
Step 6.	72°C 5 min
Step 7.	4°C hold

Check 5μL of the PCR reaction on a standard 1% agarose gel to confirm amplification success and clean negative control. Product size is approximately 550 base pairs. Digestion can be performed directly in PCR tube.

MSPI Digestion Reaction	30 μL reaction
Remaining PCR product	20.0 μL
10X NE Buffer 2	3.0 μL
10U MSP I enzyme (20U/μL)	0.1 μL
dd sterile H_2O	6.9 μL

Make a cocktail of the buffer, enzyme and water. Add 10 μL to each reaction tube containing the DNA. Incubate at 37°C overnight. Add loading dye directly to digestion reaction and load the total volume on a 3% NuSieve gel. The major bands for the AA genotype are 190 bp, 154 bp, and 136 bp; The BB genotype bands are 154 bp, 136 bp and 125 bp.

Analysis of the French lines showed that the new PCR test MSPI ALLELE A = (old) SacI Allele 2

Using the PCR-RFLP to genotype University of Nebraska high ovulating and control lines, the results by line show:

	A	B
Index	.50	.50
Control	.47	.53

Hence a higher frequency of A in the index line.

PIC Line 19 Animals (with appropriate records) analyzed with the MSP I test are shown in Tables 7 and 8 below.

Table 2.

AVE NB Genotype	N	Mean	Std Err
AA	93	10.178	0.237
AB	98	9.292	0.275
BB	18	9.722	0.511

Table 3.

EBV TNB Genotype	N	Mean	Std Err
AA	93	0.160	0.062
AB	98	0.034	0.049
BB	18	0.108	0.112

The data grouped for litter size is shown below.

	AA	AB	BB	A	B
Large Litter	.62	.36	.04	.78	.22
Small Litter	.24	.64	.12	.56	.44

EXAMPLE 4

A second PCR test was developed as indicated below.

PIC RBP4 TEST PROTOCOL

RBP4- FOR2 5'ACT GTG CTC TTT GTG CTG 3' SEQ ID NO:6

RBP4-REV 5'CTC GGT GTC TGT AAA GGT G 3' SEQ ID NO:7

Method:

10x PCT Buffer	1.2 µl
2mM dNTP's	1.2 µl
25mM Mg^{2+}	1.2 µl
RBP4-FOR 2 (5µM)	1.2 µl
RBP4-REV (5µM)	1.2 µl
Amplitag Gold	0.12 µl
QH_2O	4.88 µl
Lysate	1.0 µl

12.0 µl

PE9700

	94°C 30 secs	}
94°C 12 min	58°C 30 secs	} x30 → 72°C 4 min → 6°C
	72°C 30 Secs	}

(9600 Ramp)

Digestion: (37°C 3 Hours

PCR Product	12.0 l
MSP 1 (10u/l)	0.5 µl
QH_2O	1.0 µl
	14.0 µl

Load and run on 5% NuMe Agarose at 150 volts for approx. 45 minutes to 1 hour. Expected Band sizes are shown in Figure 1.

EXAMPLE 5

European Results

264 Landrace sows with litter size records at a PIC breeding farm in Europe were genotyped for RBP4 using the protocol of Example 4. Two alleles were identified: 1 and 2. The distribution of the marker genotypes followed Hardy Weinberg equilibrium in all lines.

Two traits were analyzed -- total number born (TNB) and numbers born alive (NBA).

Data were analyzed using a mixed model with sire as the random effect (h2=0.09).

Fixed effects included: year-month of farrowing, sowline, boarline, boar farm (3 AI studs), cycle 1, 2, 3+_, services (1, 2, 3+). All services were by AI.

Effect of RBP4 across all parities and sowlines

Genotype	N of litters	TNB	NBA
11	212	10.84	9.93
12	223	10.28	9.66
22	87	9.93	9.36
a		+.46*	+0.29
d		-.11	+0.02
*P<0.1			

Results with over 7509 litters of RBP4 suggests that the difference between homozygous genotypes is 1.05 pigs per litter.

EXAMPLE 6

154 Dam line sows consisting of Landrace and a Large White/Duroc synthetic line with litter size records at a breeding farm in the USA received genotypes for RBP4.

Genotype	N of litters	TNB
11	297	10.74
12	350	10.04
22	103	9.66
P<0.02		

All references cited supra and hereinafter are expressly incorporated in their entirety by reference:

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What is claimed is:

1. A method of screening animals to determine those more likely to exhibit increased litter size comprising: obtaining a sample of genetic material from an animal; and assaying for the presence of a polymorphism in the retinol binding protein 4 gene in said sample, said polymorphism associated with increased litter size.

2. The method of claim 1 wherein said animal is a pig.

3. The method of claim 1 wherein said step of assaying is selected from the group consisting of: direct sequence analysis, restriction fragment length polymorphism (RFLP) analysis, heteroduplex analysis, single strand conformational polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE).

4. The method of claim 1 wherein said step of assaying for the presence of said polymorphism comprises the steps of: digesting said genetic material with a restriction enzyme that cleaves said retinol binding protein 4 gene in at least one place; separating the fragments obtained from said digestion; detecting a restriction pattern generated by said fragments; and comparing said pattern with a second restriction pattern for the reproductive gene obtained by using said restriction enzyme, wherein said second restriction pattern is associated with increased litter size.

5. The method of claim 3 wherein said separation is by gel electrophoresis.

6. The method of claim 3 wherein said step of comparing said restriction patterns comprises identifying specific fragments by size and comparing the sizes of said fragments.

7. The method of claim 6 wherein said step of detecting different sizes of said fragments comprises the steps of: separating said fragments by size using gel electrophoresis in the presence of a control DNA fragment of known size; contacting said separated fragments with a probe that hybridizes with said fragments to form probe fragment complexes; and determining the size of separated fragments by detecting the presence of the probe fragment complexes and determining their relative positions with respect to said control DNA fragment.

8. The method of claim 4 further comprising the step of amplifying the amount of said reproductive gene or a portion thereof which contains said polymorphism, prior to said digestion step.

9. The method of claim 8 wherein said amplification is conducted with Taq polymerase.

10. The method of claim 8 wherein said amplification includes the steps of: selecting a forward and a reverse sequence primer capable of amplifying a region of said reproductive gene which contains a polymorphic site.

11. The method of claim 10 wherein said gene is the retinol binding protein 4 and said forward and reverse primers are selected from and based upon the sequence disclosed in Figure 1.

12. The method of claim 11 wherein said primers are TTCCGAGTCAAAGAGAACTTCG and TCATAGTCCGTGTCGATGATCC.

13. The method of claim 11 wherein said primers are GAGCAAGATGGAATGGGTT and CTCGGTGTCTGTAAAGGTG.

14. The method of claim 13 wherein said restriction enzyme is MSP I.

15. The method of claim 14 wherein said polymorphism is a 190bp restriction length polymorphism.

16. The method of claim 14 wherein said polymorphism is a 125 bp restriction length polymorphism.

17. The method of claim 16 wherein said primers are ACTGTGCTCTTTGTGCTG and CTCGGTGTCTGTAAAGGTG.

18. A primer for assaying for the presence of a polymorphic site associated with increased litter size in the porcine retinol binding protein 1 gene wherein said primer comprises at least 4 bases selected from or based upon the sequence disclosed in Figure 1.

19. The primer of claim 18 wherein said primer is selected from the group consisting of: TTCCGAGTCAAAGAGAACTTCG and TCATAGTCCGTGTCGATGATCC, GAGCAAGATGGAATGGGTT, CTCGGTGTCTGTAAAGGTG, ACTGTGCTCTTTGTGCTG, and CTCGGTGTCTGTAAAGGTG.

20. A genetic marker associated with increased litter size, said marker comprising the retinol binding protein 4 gene.

21. The marker of claim 20 wherein said marker is identifiable by a MSP I polymorphism in a region of said gene amplified by primers ACTGTGCTCTTTGTGCTG and CTCGGTGTCTGTAAAGGTG.

22. The genetic marker of claim 20 wherein said marker is identifiable by a MSP I polymorphism in a region of said gene amplified by primers GAGCAAGATGGAATGGGTT and CTCGGTGTCTGTAAAGGTG.

23. A purified and isolated DNA sequence from the porcine retinol binding protein 4 gene said sequence consisting of the sequence depicted in Figure 1.

24. A method for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce smaller litters, which method comprises of the steps of: determining an allele of a retinol binding protein 4 gene present in an animal; determining an allele of other markers for genes known to affect litter size; and selecting for animals with favorable combinations of alleles and against those carrying unfavorable combinations.

25. The method of claim 24 wherein the determination of reproductive gene alleles comprises: determining the presence of at least one allele associated with at least one DNA marker linked either directly or indirectly to said reginol binding protein 4 gene.

26. The method as claimed in claim 25 wherein the DNA marker is a microsatellite.

27. A method of screening animals to determine those more likely to have larger litters comprising: obtaining a sample of genetic material from said animal; and assaying for the presence of a polymorphism in the retinol binding protein 4 gene said polymorphism being one which is identifiable by the presence or absence of a MSP I polymorphism in the region amplified by the primers ACTGTGCTCTTTGTGCTG and CTCGGTGTCTGTAAAGGTG.

28. A method of screening animals to identify polymorphic markers linked to specific reproductive genes which are associated with altered litter size comprising: selecting nucleic acid sequences linked to the reproductive gene RBP4; obtaining nucleic acid from individual animals differing in their breeding value for reproductive traits; assaying for polymorphisms in said genes; and associating such polymorphisms with high or low breeding value.

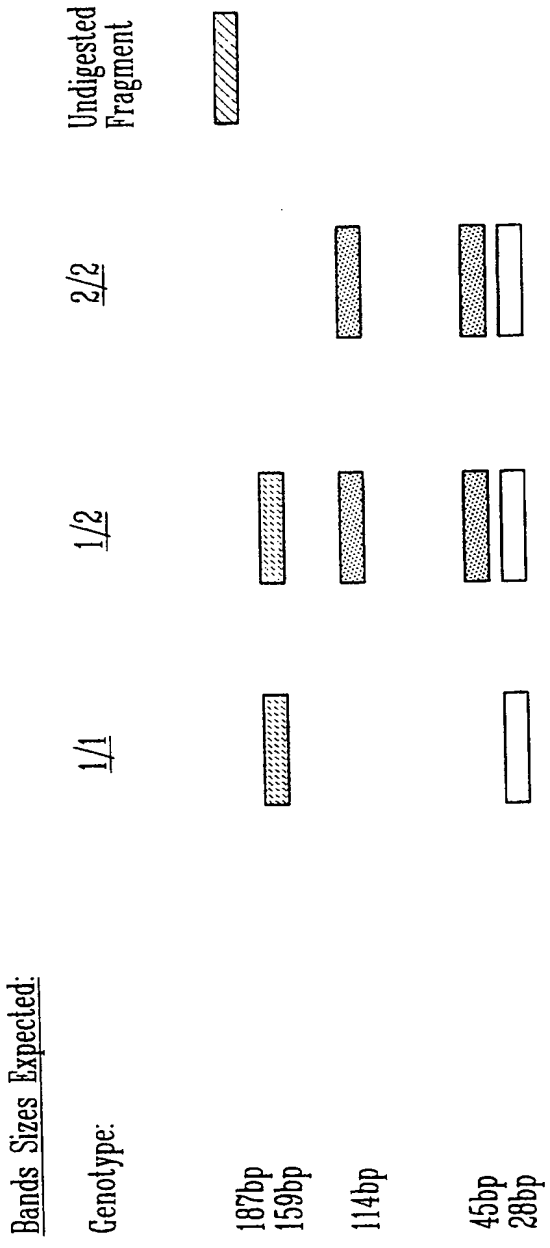

Fig. 1

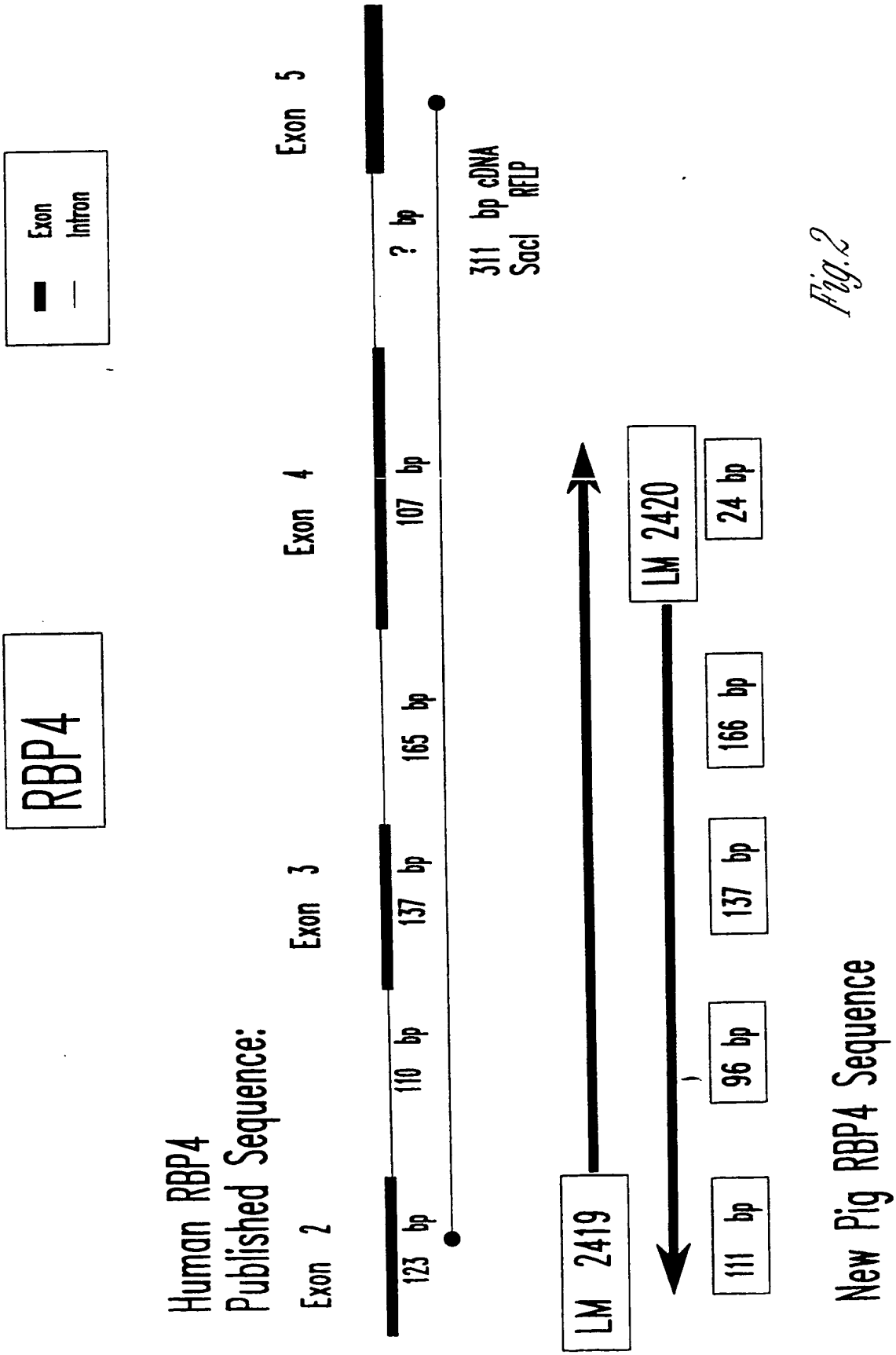

Fig.2

INTERNATIONAL SEARCH REPORT

Inter ional Application No
PCT/US 99/00866

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MESSER, L. ET AL.: "Mapping and investigation of candidate genes for litter size in french white pigs" ANIMAL GENETICS, vol. 27, no. suppl 2, December 1996 (1996-12), page 114, ABS E056 XP002115295 the whole document ---	1-7,20, 24,25,28
A	MESSER, L. ET AL.: "Linkage mapping of the retinol-binding protein 4 (RBP4) gene to porcine chromosome 14" MAMMALIAN GENOME, vol. 7, no. 5, 1996, page 396 XP002115296 the whole document --- -/--	1-7,24, 25,28

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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Date of the actual completion of the international search	Date of mailing of the international search report
16 September 1999	28/09/1999

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Inter onal Application No
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